

NEUROBLASTOMA CELL LINES EXPRESSING THE $\alpha 2\delta$ SUBUNIT OF CALCIUM CHANNELS AND METHODS THEREFORE

Field of the invention

5 The present invention relates to neuroblastoma cells that express the $\alpha 2\delta$ subunit of the calcium channel. In another aspect of this invention, the invention relates to methods and assays using neuroblastoma cells and neuroblastoma cell membranes. The cells of the present invention are useful for discovering new compounds that modulate the function of the $\alpha 2\delta$ subunit of calcium channel.

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Background of the invention

Several subtypes of the $\alpha 2\delta$ subunit of calcium channel have been cloned (Angeloni et al. *Mol. Cell. Probes* 14:53-54, 2000; Gao et al., *J. Biol. Chem.* 275:12237-12242, 2000; and PCT Application WO 99/23519).

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Neuroblastoma cell membranes contain N-type calcium channels and neuroblastoma cells have been used as a model for neuronal differentiation (Bruhn, et al. *Endocrinology* 137:572-9, 1996; Gotti, et al. *Differentiation (Berlin)* 34:144-55, 1987; Hogg et al. *Pharmacol.*, 312:257-261, 1996; and Kurata, et al. *FEBS Lett.*, 321:201-4, 1993). 5-Bromo-2'-deoxyuridine (BrdU) induces morphological and functional differentiation of neuroblastoma cells, resulting in an increase of neurotransmitter receptors and the release of neurotransmitters (Clementi, et al. *Adv. Exp. Med. Biol.* 296:91-102, 1991). [125 I] ω -conotoxin binding sites were increased in the differentiated neuroblastoma IMR32 cells, indicating that the N-type calcium

channels were increased in the cells (Carbone et al., *Pfluegers Arch.*, 416:170-9, 1990). Recently, Western blot analysis has shown that the $\beta 1b$ subunit of calcium channels is the predominant isoform expressed in IMR32 cells (McEnery, et al. *FEBS Lett.* 420: 74-78, 1997).

- 5 Gabapentin (GBP) is an anticonvulsant that has shown usefulness in the treatment of neuropathic pain (Backonjy, M. et al. *J. Am. Med. Assoc.* 280:1831-1836, 1998; Laird and Gidal, *Ann. Pharmacother.* 34:802-807, 2000; and Rowbotham et al. M., *J. Am. Med. Assoc.*, 280:1837-1842, 1998). GBP inhibits neurotransmitter release (Dooley, et al. *Neurosci.Lett.* 280:107-110, 2000) and
- 10 inhibits calcium currents in brain neurons (Fink, et al. *Br. J. Pharmacol.* 130:900-906, 2000; Larid and Gidal, *supra*; and Stefani et al. *Neuropharmacology* 37:83-91, 1998). Interestingly, a high-affinity binding site for GBP was found in brain tissue and the target protein was identified as the $\alpha 2\delta$ subunit of subunit of calcium
- 15 channels (Brown and Gee, *J. Biol. Chem.*, 273:25458-25465, 1998; Dissanayake, et al. *Br. J. Pharmacol.*, 120:833-840, 1997; and Gee et al., *J. Biol. Chem.* 271:5768-76, 1996). An autoradiographic binding study showed that the GBP binding site was widely distributed in rat brain areas such as frontal cortex, striatum, hippocampus and cerebellum (Hill and Woodruff, *Eur. J. Pharmacol. Mol. Pharmacol. Sect.*, 244:303-9, 1993; Thurlow et al. *Br. J. Pharmacol.*, 118:457-465,
- 20 1996). Therefore compounds that compete with Gabapentin binding to the $\alpha 2\delta$ subunit should be useful as anticonvulsants and in the treatment of neuropathic and chronic pain.

Summary of the invention

The present invention relates to a method for detecting binding of a test substance to an $\alpha 2\delta$ subunit comprising the steps of: contacting a neuroblastoma cell membrane sample comprising the $\alpha 2\delta$ subunit with gabapentin and a test substance; detecting binding of gabapentin to the cell membrane; and, comparing the level of binding of gabapentin as compared with a control sample lacking the test substance.

In one embodiment the cell membranes are part of intact cells and in another embodiment the cell membranes are obtained from isolated cell membrane preparations. In one embodiment the cells are the neuroblastoma cells IMR32. Preferably the neuroblastoma cell membranes are differentiated neuroblastoma cell membranes and in one embodiment the differentiated cell membranes are obtained following cell incubation with BrdU.

In a preferred method, the methods of this invention further comprise the step of separating the cell membranes from unbound gabapentin. In another preferred method, the comparing step comprises measuring binding of labeled gabapentin bound to the cell membranes. The invention further relates to compounds identified using the methods of this invention.

In another embodiment the invention further relates to a test substance identified by a method comprising the steps of: contacting a neuroblastoma cell membrane sample comprising the $\alpha 2\delta$ subunit of a calcium channel with gabapentin and a test substance; detecting binding of the gabapentin to the cell membrane; and comparing the level of binding of gabapentin as compared with a control sample lacking the test substance.

The invention further relates to a method for identifying a test substance capable of binding to an $\alpha 2\delta$ subunit of a calcium channel comprising the steps of: incubating an IMR32 cell membrane with radioactive gabapentin (GBP) and a test substance, wherein the membrane comprises an $\alpha 2\delta$ subunit of calcium channel and
5 where the contact is for sufficient time to allow GBP binding to the $\alpha 2\delta$ subunit of calcium channels in the cell membranes; separating the cell membranes from unbound radioactive GBP; measuring binding of the radioactive GBP to the cell membranes; and identifying a compound that inhibits GBP binding by a reduction of the amount of radioactive GBP in the measuring step to an established control.

10 The methods of the present invention are useful for identifying compounds that interact with the $\alpha 2\delta$ subunit of calcium channels. Compounds identified using the methods of the present invention can then be tested for their ability to treat biological conditions mediated by the $\alpha 2\delta$ subunit of calcium channels.

15 **Brief description of the drawings**

Fig. 1. Effect of membrane protein concentration on [^3H]GBP binding. The membranes of human neuroblastoma IMR32 cells were incubated with [^3H]GBP (10 nM) at 25°C for 60 min. The data are representative of two experiments with each point assayed in duplicate. Black squares = Total bound counts; Clear squares =

20 Specific binding; Clear triangles = nonspecific binding.

Fig. 2. Saturation binding of [^3H]GBP to IMR32 cell membranes. (A) Varying concentrations of [^3H]GBP were incubated with membranes (40 μg protein/ml)

from non-differentiated IMR32 cells. Black squares = Total bound counts; Black circles = Specific binding; Clear squares = nonspecific binding.

(B) Varying concentrations of [^3H]GBP were incubated with membranes (40 μg protein/ml) from IMR32 cells differentiated by 10 μM BrdU. Black squares = Total bound counts; Black circles = Specific binding; Clear squares = nonspecific binding.

(C) Scatchard plot from the data of Fig. 2A and Fig. 2B. Clear squares = differentiated IMR32 cells; Black squares = nondifferentiated IMR32 cells

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Fig. 3. Inhibition of [^3H]GBP binding to human neuroblastoma IMR32 cell membranes by GBP and L-methionine. Varying concentrations of unlabeled GBP and L-methionine were incubated with membranes (20 μg protein/ml) of non-differentiated and differentiated IMR32 cells in the presence of 10 nM [^3H]GBP.

15 The results shown represent two experiments with each point assayed in duplicate. Black squares = GBP binding to nondifferentiated IMR32 cells; Black triangles = L-methionine binding to nondifferentiated IMR32 cells; Clear squares = GBP binding to differentiated IMR32 cells; Clear triangles = L-methionine binding to differentiated IMR32 cells

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Detailed description of the invention

The present invention provides methods to identify compounds that modulate the function of the $\alpha 2\delta$ subunit of the calcium channel in neuroblastoma cells and preferably in differentiated neuroblastoma cells.

5 There are a variety of neuroblastoma primary cells and cell lines that can be used in the methods of this invention. For purposes of this invention the term "neuroblastoma cell lines" includes cell lines, cells isolated from tumor explants and cell hybrids prepared by fusion of a cell with a neuroblastoma cells. Preferred neuroblastoma cells of the present invention are of human origin but can also be of
10 animal origin. Exemplary cells and cells lines include, but are not limited to, IMR32 cells, SK-N-MC cells and NG 108 cells (a mouse neuroblastoma/rat glioma hybrid cell line). These cells are available from a number of sources including the ATCC (Manassas, VA). While established cell lines are preferred, cells useful for this invention can also be isolated from a variety of vertebrate sources, such as
15 animal or human tumor explants.

 In a preferred embodiment, IMR32 cells are used and in another preferred embodiment the neuroblastoma cells are exposed to a differentiation-promoting agent, such as BrdU. Other differentiation-promoting agents that can be used in this invention include, but are not limited to, dibutryl cyclic AMP, neural growth factor
20 (NGF) and retinoic acid. Differentiated neuroblastoma cells tend to express increased levels of $\alpha 2\delta$ subunit protein as compared with undifferentiated cells.

 There are a number of methods known in the literature for differentiating undifferentiated neuronal cells, such as neuroblastoma cells, and an exemplary method is found in the example section, provided below. In general, however, the

cells are exposed to a non-cytotoxic amount of the differentiation-promoting agent for a time sufficient to induce differentiation in the cell culture. Differentiation can be determined visually, through the outgrowth of neural processes, for example, or in the expression of a more differentiated phenotype, including increased adherence to the substrate, improved growth control and a general flattening of the cell morphology. Alternatively, cell differentiation can be determined by detecting proteins that are known to be associated with a more differentiated cell phenotype, as is known in the art.

The assays of this invention can be used as intact cell assays or the assays can be performed using membrane lysates or purified membrane preparations from cells expressing the calcium channel $\alpha 2\delta$ subunit. Where cells membranes are used, the $\alpha 2\delta$ subunit of calcium channels is isolated as a component of the neuroblastoma cell membranes. Isolated cell membranes are prepared using conventional means such as homogenizing the cells via mechanical force.

Gabapentin (GBP) (CAS number 60142-96-3) is used as a specific ligand for the $\alpha 2\delta$ subunit of calcium channels. In a preferred embodiment the GBP is labeled to facilitate detecting GBP binding to the $\alpha 2\delta$ subunit. GBP can be labeled using any number of methods known in the art including fluorescent labels, radioactive labels, and the like. In a preferred embodiment, a commercially available [^3H]GBP is used as a detectable ligand in the displacement assays of the present invention.

In the assays of the present invention, the cell membranes are combined with GBP and a test substance. The test substance can be any candidate molecule that

one hypothesizes will bind to the $\alpha 2\delta$ subunit. These include small molecules, peptides, polypeptides, including antibodies, and the like.

The cell membrane mixture comprising GBP and the test substance are then incubated in an aqueous buffer for a time sufficient to permit the GBP to bind to the $\alpha 2\delta$ subunit contained in the cell membranes. The amount of incubation time
5 necessary depends on the amount of reagents used, temperature, and other factors. Varying the reaction conditions using methods well known in the art alters the amount of label incorporation into the cell membranes and these conditions can be readily optimized by those of ordinary skill in the art. After incubation the cell
10 membranes are isolated from unbound GBP using conventional means, including filtration or centrifugation.

The ability of the test substance to bind to the $\alpha 2\delta$ subunit is determined by measuring a reduction in the amount of GBP binding to the cell membranes in samples containing both GBP and test substance as compared to a control reaction
15 that does not include the test substance and which preferably includes both unlabeled and labeled GBP. Where the GBP is radiolabeled, the level of radioactivity in treated cell membranes compared to control membranes is measured.

Candidate compounds that are capable of competing with GBP for binding
20 are identified using the methods of the present invention. These compounds can then be tested for their ability to affect a number of biological conditions mediated by the $\alpha 2\delta$ subunit of calcium channels.

The present invention is exemplified by way of the following examples.
These examples are not intended to limit the present invention.

Example 1

5 **Differentiation of IMR32 cells and analysis of the expression of the $\alpha 2\delta$ subunit of calcium channels**

Materials and Methods

IMR32 cells, SK-N-MC cells and NG 108 cells as well as cells treated in
culture with 10 μ M of BrdU for 10 to 12 days were used. Cells were harvested and
10 homogenates were made in HEPES/KOH buffer, pH 7.4. The homogenates were
centrifuged for 15 min at 1000 x g, and the supernatants were centrifuged at 40000 x
g for 15 min to obtain cell membranes.

A [3 H]GBP binding assay was performed as described by Gee, et al. (*supra*).

Non-specific binding was defined in the presence of 100 μ M unlabeled GBP.

15 Separation of bound from free ligand was effected by filtration through 0.3%
polyethylenimine-soaked GF/B filters. The filters were washed with 3 x 4 ml of 10
mM HEPES pH 7.4. Radioactivity on filters was determined by scintillation
counter.

[3 H]GBP binding increased with increasing concentrations of cell
20 membranes. [3 H]GBP bound to cell membranes from all neuroblastoma cell lines
tested. The increase in binding was linear with membrane concentrations up to 100
 μ g protein /ml (Fig. 1).

In one example to test the effect of BrdU on the density of [^3H]GBP binding sites in the differentiated IMR32 cells, saturation experiments were performed using both un-differentiated and differentiated IMR32 cell membranes (Fig. 2). Scatchard analysis of the saturation binding data suggests a single high affinity GBP binding site on IMR 32 cell membranes with a K_d value of 37 nM and B_{\max} value of 1186 fmol/mg protein. BrdU increased the expression of [^3H]GBP binding sites (B_{\max} = 2245 fmol/mg protein) without changing its affinity (K_d = 39 nM).

The pharmacology of [^3H]GBP binding sites was examined by investigating the ability of unlabeled GBP and L-methionine to inhibit binding. 10 nM of [^3H]GBP was used for the experiments. The K_i values were obtained using a one-site binding model and are summarized in Table 1. Fig. 3 shows that unlabeled GBP and L-methionine dose-dependently inhibited [^3H]GBP binding to IMR32 cell membranes. BrdU did not significantly alter the K_i values of GBP and L-methionine for the cell membranes (Table 1).

Table 1. K_i values of GBP and L-methionine inhibition of [3 H]GBP binding to undifferentiated (Control) and differentiated (BrdU) IMR32 cell membranes.

	K_i (nM)	
	Control	BrdU
GBP	29	15
L-methionine	39	30

The increase in [3 H]GBP binding by BrdU in neuroblastoma cells suggests that the $\alpha 2\delta$ subunit of calcium channels increases during the differentiation of human neuroblastoma cells. This increase, in addition to previous results, which indicated that the $\alpha 1$ and $\beta 1b$ subunits were also increased by BrdU (Carbone, et al., *supra* and McEnery et al., *supra*), suggests that most of the major subunits of calcium channels are up-regulated during the differentiation of human neuroblastoma cells. Full activation of calcium channels requires both β and $\alpha 2\delta$ subunits (Shistik, et al. *J. Physiol. (London)*:489:55-62, 1995 and Walker and DeWaard, *Trends Neurosci.*, 21:148-154, 1998). The present results imply that calcium channels play an important role in the differentiation of human neuroblastoma cells.

Several L-amino acids, such as leucine, methionine, phenylalanine and valine, inhibit [3 H]GBP binding to synaptic plasma membranes. L-methionine is one of the most potent inhibitors (Thurlow, et al., *Eur. J. Pharmacol. Mol. Pharmacol. Sect.*, 247:341-5, 1993). We found that GBP and L-methionine inhibited [3 H]GBP binding to IMR32 cell membranes. The K_i values of GBP and

L-methionine were not significantly different between undifferentiated and differentiated cell membranes, indicating that BrdU did not change the affinities of GBP and L-methionine for cell membrane binding.

5 GBP binding to calcium channels requires both $\alpha 2$ and δ subunits (Wang, et al. *Biochem. J.*, 342:313-320, 1999). The region between the N-terminal end and the first transmembrane domain of $\alpha 2$, as well as the region between the splicing acceptor sites may play important roles in maintaining the structural integrity for GBP binding.